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ORIGINAL PAPER

Properties of a purified thermostable glucoamylase from *Aspergillus niveus*

Tony Marcio da Silva · Alexandre Maller · André Ricardo de Lima Damásio · Michele Michelin · Richard John Ward · Izaura Yoshico Hirata · João Atilio Jorge · Héctor Francisco Terenzi · Maria Lourdes T. M. de Polizeli

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Abstract A glucoamylase from *Aspergillus niveus* was produced by submerged fermentation in Khanna medium, initial pH 6.5 for 72 h, at 40°C. The enzyme was purified by DEAE-Fractogel and Concanavalin A-Sepharose chromatography. The enzyme showed 11% carbohydrate content, an isoelectric point of 3.8 and a molecular mass of 77 and 76 kDa estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis or Bio-Sil-Sec-400 gel filtration, respectively. The pH optimum was 5.0–5.5, and the enzyme remained stable for at least 2 h in the pH range of 4.0–9.5. The temperature optimum was 65°C and retained 100% activity after 240 min at 60°C. The glucoamylase remained completely active in the presence of 10% methanol and acetone. After 120 min hydrolysis of starch, glucose was the unique product formed, confirming that the enzyme was a glucoamylase (1,4-alpha-D-glucan glucohydrolase). The K_m was calculated as 0.32 mg ml^{-1}. Circular dichroism spectroscopy estimated a secondary structure content of 33% α-helix, 17% β-sheet and 50% random structure, which is similar to that observed in the crystal structures of glucoamylases from other *Aspergillus* species. The tryptic peptide sequence analysis showed similarity with glucoamylases from *A. niger, A. kawachi, A. ficcum, A. terreus, A. awamori* and *A. shirousami*. We conclude that the reported properties, such as solvent, pH and temperature stabilities, make *A. niveus* glucoamylase a potentially attractive enzyme for biotechnological applications.

Keywords Glucoamylase · Fungi · Thermostability · Organic solvents · *Aspergillus niveus*

Introduction

Amylases are enzymes present in many organisms. In plants they participate in the synthesis of starch in some types of roots and in animals for the digestion of starch in food, and are found in many prokaryotic and eukaryotic microbes that use starch as a carbon source [28]. Glucoamylases hydrolyze α-1,4 and α-1,6 linkages of starch and related polymers to produce glucose as the sole end product. Glucoamylases also hydrolyze other starch-related oligo- and polysaccharides, and show a preference for maltooligosaccharides of at least six residues [22]. One of most important applications of glucoamylases is the production of high glucose syrups from starch, and these enzymes are also used in the production of ethanol and in the baking and brewing industries [35].

Industrial applications require enzymes with high activity and thermostability, and for the starch processing

T. M. da Silva · M. Michelin · J. A. Jorge · H. F. Terenzi · M. L. T. M. de Polizeli (✉)
Departamento de Biologia, Faculdade de Filosofia Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, Av. Bandeirantes 3900, Monte Alegre, 14040-901 Ribeirão Preto, São Paulo, Brazil
e-mail: polizeli@ffclrp.usp.br

A. Maller · A. R. de Lima Damásio
Departamento de Bioquímica e Imunologia, FMRP, Universidade de São Paulo, Ribeirão Preto, São Paulo, Brazil

R. J. Ward
Departamento de Química, FFCLRP, Universidade de São Paulo, Ribeirão Preto, São Paulo, Brazil

I. Y. Hirata
Departamento de Biofísica, Universidade Federal de São Paulo, São Paulo, SP, Brazil

industry there are many advantages of using thermostable enzymes, such as an increased reaction rate and decreased contamination risk through the use of high temperatures [14, 15]. Several microbial strains are known sources of glucoamylase production, and several species of *Aspergillus* and *Rhizopus* have been widely used for commercial production [12, 33].

Enzymes with high stability and activity in the presence of organic solvents would be useful for biotechnological applications in which these solvents are used. Organic-solvent-tolerant prokaryotic microorganisms have been studied [7, 8, 10, 26, 27]; however, fungal enzymes with stability and activity in the presence of organic solvents have not yet been reported. The aim of this work was to describe biochemical properties of a glucoamylase purified from the thermotolerant filamentous fungus *A. niveus*, which presents high amylolytic activity in the presence of organic solvents and elevated thermostability.

Materials and methods

Microorganism and growth conditions

Aspergillus niveus was isolated from *Mangifera indica* in our laboratory. The microorganism was identified and deposited in the culture collection of the University Recife Mycology—URM (PE, Brazil), WFCC, number 604. The organism was maintained on slants of PDA medium covered with mineral oil and stored at 4°C. Approximately 10^7 conidia/ml from 3-day-old cultures were inoculated into 125-ml Erlenmeyer flasks containing 25 ml of modified Khanna medium [16] (0.1% yeast extract, 5% Khanna salt solution (2% NH_4NO_3, 1.3% KH_2PO_4, 0.36% $MgSO_4{\cdot}7H_2O$, 0.1% KCl, 0.07% $ZnSO_4{\cdot}H_2O$, 0.014% $MnSO_4{\cdot}H_2O$, 0.007% $Fe_2(SO_4)_3{\cdot}6H_2O$, 0.006% $CuSO_4{\cdot}5H_2O$) and 1% starch. After inoculation, the cultures were maintained at 40°C for 72 h under static conditions at an initial pH 6.5. After filtration of the culture in a Büchner funnel and filter paper Whatman® no. 1, the filtrate was used as a source of crude extracellular amylolytic activity.

Determination of amylase activity

The activity of glucoamylase was routinely assayed with 1% soluble commercial starch in 100 mM sodium acetate buffer, pH 5.0, and the released reducing sugar was determined by the Miller method [24]. One enzyme unit was defined as the amount that releases reducing sugar at an initial rate of 1 μmol min^{-1} at the temperature optimum of the enzyme (in this case 65°C, see "Results and discussion"). Extracellular protein was determined by the Lowry method [21] using bovine serum albumin as standard.

PAGE of purified glucoamylase

The purified sample was applied in 6% PAGE [5], after the gel was incubated in 500 mM sodium acetate buffer, pH 5.0, for 1 h, at 4°C for determination of the glucoamylase activity. The gel was subsequently immersed in 1% starch solution for 30 min at 60°C and developed with 0.1% I_2 + 1.5% KI. In parallel, a separate gel was run under identical conditions and developed with $AgNO_3$ for protein visualization.

Glucoamylase purification

All steps were carried out at 4°C. The crude enzyme extract was applied to a DEAE-Fractogel TSK 650M column (2.0 × 7.0 cm) previously equilibrated in 10 mM Tris–HCl buffer pH 7.5 and eluted at a flow rate of 100 ml/h. Fractions of 10 ml were collected and assayed for enzyme activity. The pool showing amylase activity was applied to a Concanavalin A-Sepharose affinity chromatographic column (1.2 × 5.0 cm), and a 1-ml fraction was collected. The glucoamylase was eluted with a linear gradient of methyl α-D-mannopyranoside (0–0.3 M, 50 ml) in Tris–HCl buffer containing 500 mM NaCl, 0.5 mM $MnCl_2$ and 0.5 mM $CaCl_2$, at a flow rate of 36 ml/h. Protein elution was monitored by measurement of absorbance at 280 nm.

SDS-PAGE of purified glucoamylase

Protein homogeneity and the molecular mass of the enzyme were evaluated by 6% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously by Laemmli [18]. Gels were stained with 0.25% Coomassie Brilliant Blue R-250 and destained with methanol-acetic acid-water (3:1:6). Molecular mass markers were: myosin (205 kDa), phosphorylase (97 kDa), bovine serum albumin (66 kDa) and ovalbumin (45 kDa).

Deglycosylation assay

Deglycosylation of the glucoamylase was performed using the enzymes Endo H (Endo-*β*-*N*-acetilglucosaminidase H; EC 3.2.1.96) from *Streptomyces plicatus* or PNGase F (Peptide-*N*-glycosidase F; EC 3.2.1.18) from *Flavobacterium meningosepticum*. One unit of each enzyme was used to deglycosylate 50 μg of purified glucoamylase at 37°C for 48 h. Samples were analyzed by SDS-PAGE.

Enzymatic characterization

The pH optimum was determined at 60°C using citrate-phosphate buffer (pH range 3.0–7.0). The pH stability was determined at 30°C, for 2 h, after pre-incubation of the

diluted enzyme in citrate phosphate buffer at different pH values (pH range 3.0–9.0). The temperature optimum was determined with 0.1 M sodium acetate buffer, pH 5.5. The thermostability was determined by measuring the residual activity after incubation of the diluted enzyme in the absence of substrate at 60°C in 0.1 M sodium acetate buffer, pH 5.0, for 6 h. For determination of the pH and temperature stabilities, the enzymatic assays were carried out using 1% soluble starch as substrate. For the determination of the effect of metallic ions on glucoamylase activity, assays were performed in 1 or 10 mM final concentration of metallic ions, where the activity in the absence of metallic ions was defined as the control. For determination of the effect of organic solvents, the enzymatic assay was performed after pre-incubating the enzyme with 5 or 10% final concentration of solvents. The activity assayed in the absence of solvents was defined as the control. The K_m and V_{max} values for the purified enzyme were determined by incubating the enzyme with 0–300 mg soluble starch in 100 mM sodium acetate buffer, pH 5.5, at 65°C. The data obtained were fitted to a standard Hanes model using linear regression [20].

Identification of hydrolysis products

The hydrolysis products of the amylase activity against soluble 1% starch as substrate were analyzed by thin-layer chromatography. The mobile phase was butanol/ethanol/water (5:3:2). Sugars were determined with 0.2% orcinol in a 9:1 methanol–sulfuric acid mixture [4]. Glucose, maltose, maltotriose, maltotetraose and maltopentaose were used as standards.

Dichroism circular and amino acid sequence analysis

Circular dichroism measurements were performed in 20 mM HEPES pH 7.0, at 25°C. Far ultraviolet circular dichroism spectra (190–250 nm) were measured with a JASCO 810 (JASCO Inc., Tokyo, Japan) using 1-mm path length cuvettes and a protein concentration of 150 µg/ml protein. A total of nine spectra were collected, averaged and corrected by subtraction of a buffer blank. The secondary structure content of the protein was estimated using the program K2D [3] as implemented in the DicroProt suite of programs [6]. Molar ellipticity calculations assumed that the protein shares high sequence similarity with the glucoamylases from other *Aspergillus* species and therefore is comprised of 614 amino acids. The tryptic amino acid sequence analysis of purified glucoamylase was made using the automatic sequencing machine PPSQ/23 (Shimadzu Corp., Tokyo, Japan) with isocratic system HPLC. The information obtained served as the basis for comparison with glucoamylases of other species of the genus *Aspergillus*.

Results and discussion

Purification of the amylolytic enzyme

Glucoamylase from culture filtrate was purified by two chromatographic steps (Table 1). Crude filtrate (150 ml) containing the glucoamylase was applied to a DEAE—Fractogel column equilibrated with 10 mM Tris–HCl buffer, pH 7.5, and the enzyme activity was eluted in the same buffer. This initial step resulted in the exclusion of approximately 42% of protein contaminants from the crude filtrate. The eluted fractions containing the amylase activity were pooled and applied to a Concanavalin A-Sepharose affinity column, and amylolytic activity was eluted with a linear gradient of (0–0.3 M) methyl α-D-mannopyranoside, resulting in a 2.53-fold purification with a recovery of 52%. The purification protocol resulted in a single band with amylolytic activity on solubilized starch PAGE, and a single protein band was observed with SDS-PAGE (Fig. 1a, b, lanes 1–3, respectively).

Biochemical characterization

The molecular mass of the purified glucoamylase was 77 kDa by SDS-PAGE and 76 kDa by gel filtration on Bio-Sil-Sec-400 (Bio Rad). The purified enzyme bound to Concanavalin A Sepharose, suggesting that it was a glycoprotein with a sugar content of approximately 11%. After PNGase F treatment, the mobility of protein was modified on SDS-PAGE, demonstrating a molecular mass of 68 kDa (Fig. 1b, lane 3). The carbohydrate moiety in some glycoproteins produced by filamentous fungi is responsible by

Table 1 Purification of extracellular glucoamylase from *Aspergillus niveus*

Step	Protein (total mg)	Activity (total U)	Specific activity (U/mg protein)	Yield (%)	Purification fold
Crude extract	19.0 ± 1.3	4,500 ± 97	237	100	1.0
DEAE-Fractogel	11.0 ± 0.9	3,920 ± 66	356	87.1	1.5
Con A-Sepharose	3.9 ± 0.4	2,340 ± 51	600	52.0	2.53

Results are mean values of three replicates

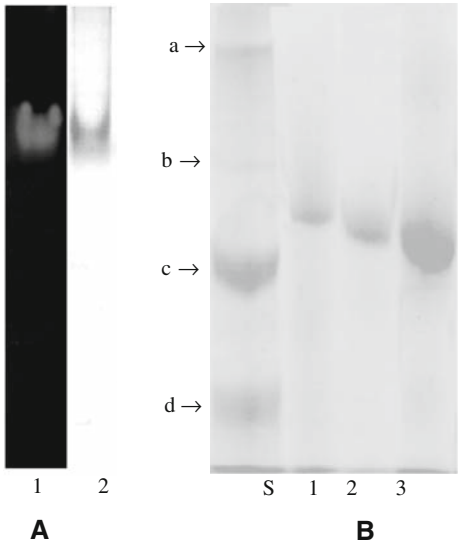

Fig. 1 Polyacrylamide gel electrophoresis and SDS-PAGE of purified glucoamylase. **a** PAGE of glycosylated GA: (*lane A1*) GA activity; (*lane A2*) GA developed by $AgNO_3$. **b** SDS-PAGE of GA stained by Comassie Blue: (*S*) standards of molecular mass (*a* myosin, *b* phosphorylase, *c* bovine serum albumin and *d* ovoalbumin); (*lane B1*) glycosylated GA, (*lane B2*) deglycosylated GA after treatment with Endo-H; (*lane B3*) deglycosylated GA after treatment with PNGase F

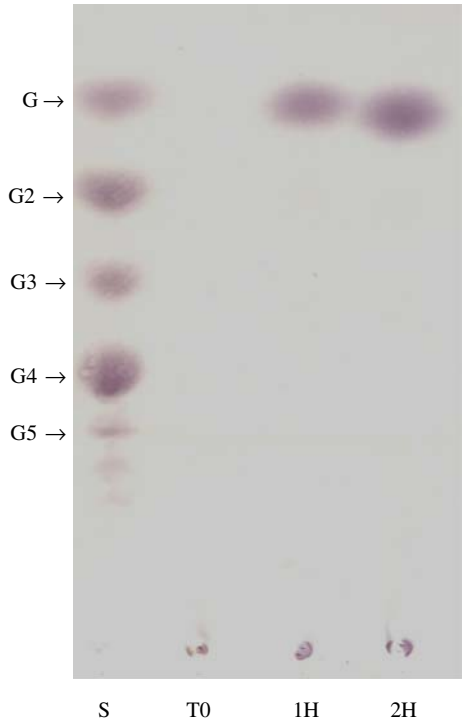

Fig. 2 Thin layer chromatography analysis of the end products of hydrolysis of purified GA under soluble starch: *S* standard (*G* glucose, *G2* maltose, *G3* maltotriose, *G4* maltotetraose and *G5* maltopentaose); *T0* zero time of assay; *1H* 1 h of hydrolysis and *2H* 2 h of hydrolysis

stability against thermal and chemical denaturation [13]. Glucoamylase with glycoproteic structures is well documented for several filamentous fungi, where the carbohydrate content is typically in the range of 10–20%. An exception appears to be the glucoamylase from *Neurospora crassa*, which has a reduced carbohydrate content of 5.1% [32]. The molecular masses of glucoamylases produced by filamentous fungi appear to vary considerably, not only among organisms, but also within different strains of the same species. The molecular masses of glucoamylases from several fungi are generally between 48 and 90 kDa [25], although exceptions, such as the 125-kDa glucoamylase produced by *A. niger*, have been reported [34]. The reduction of molecular mass of *A. niveus* glucoamylase observed after deglycosylation showed that the enzyme is a glycoprotein; however, further study is required to determine the structure and the role of the carbohydrate moiety.

The enzyme showed an excellent capacity for hydrolyzing different substrates and exhibited high affinity for soluble starch, glycogen, amylopectin, maltose, maltotriose and amylose (data not shown). The ability to hydrolyze long-chain as starches and glycogen indicated that the enzyme was a glucoamylase. On the other hand, the lack of activity on α-PNPG and sucrose indicated that the enzyme was not an α-glucosidase. Additional evidence in favor of the amylolytic activity produced by *A. niveus* being a glucoamylase was the exclusive liberation of glucose as the end product from starch hydrolysis (Fig. 2).

The effect of pH on the enzyme activity is shown in Fig. 3a. The optimum for the enzyme activity was observed in pH 5.0–5.5, and the isoelectric point is 3.8 (see Table 2). Similar results were observed by Michelin et al. [23]. The acid pH optimum is similar to other fungal glucoamylases, for example, the glucoamylase produced by two lineages of *A. terreus*, which presented pH optimum of 4.0 and 5.0 [2, 11], respectively. The glucoamylase activity of *A. niveus* was acid-alkaline tolerant and remained stable over a pH range of 4.0–9.5 after 2 h incubation (Fig. 3b). The temperature optimum of the glucoamylase from *A. niveus* was 65°C (Fig. 3c), and maintained total activity after 240 min incubation at 60°C (data not shown). The enzyme presented an elevated temperature optimum and good thermostability, which are both substantially superior to the enzyme of *Aspergillus* sp AS-2 (50°C) [30]. This result reinforces the potential of this enzyme for applications in those processes that demand elevated temperatures. The kinetic analysis using soluble starch as substrate yielded a K_m value of 0.32 ± 0.05 mg ml^{-1} (Table 2). The enzyme exhibited high affinity for starch, presenting a K_m that was lower than that reported for the glucoamylase from *A. niger* (1.48 mg ml^{-1}) [19].

Many fungal amylases described in the literature are activated by metal ions, and the glucoamylase from *A. niveus* showed a slight activity increase in the presence of low

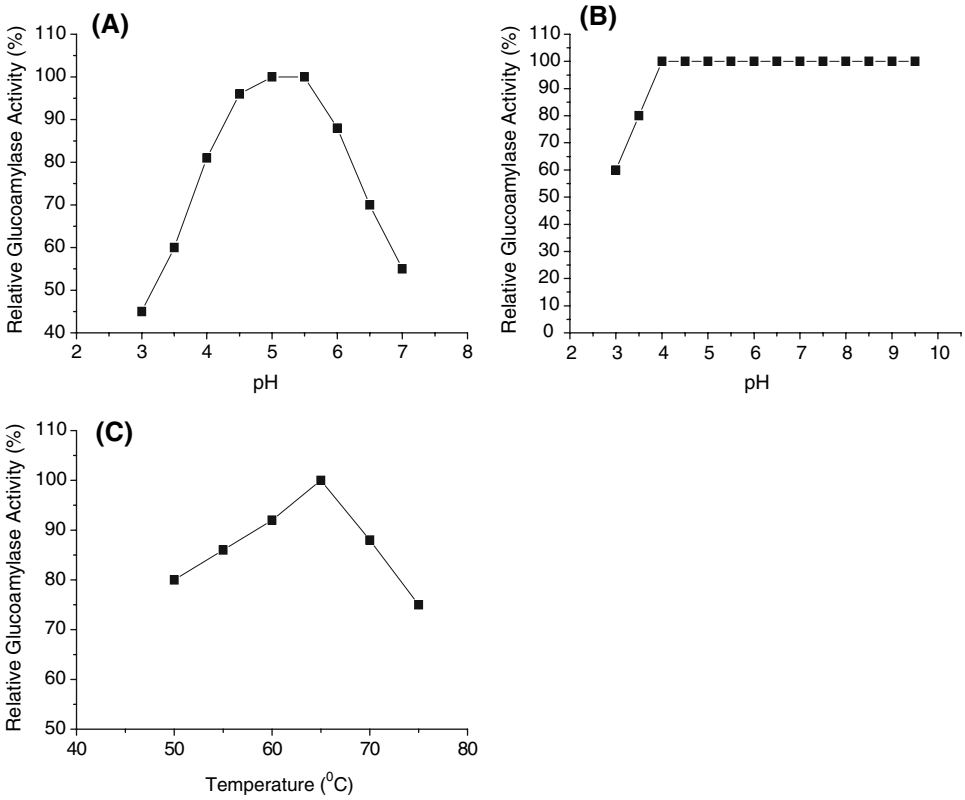

Fig. 3 Effect of pH and temperature in the glucoamylase activity and stability. **a** Effect of pH, **b** enzyme stability as a function of pH and **c** effect of temperature

Table 2 Biochemical and physical–chemical properties of the glucoamylase purified from *A. niveus*

Properties	Values
Molecular mass	
SDS-PAGE glycosylated glucoamylase	77.0 ± 0.5
SDS-PAGE enzyme-treated PNGase F	68.0 ± 0.6
Gel filtration	76 ± 0.2
Optimum pH	5.0–5.5
pH, stability 2 h	4.0–9.5
Optimum temperature (°C)	65
Temperature stability—4 h at 60°C (%)	100
K_m (mg ml^{-1})	0.32 ± 0.05
V_{max} (U/mg protein)	237.0 ± 25
K_{cat} (s^{-1})	14.2
K_{cat}/K_m (s^{-1} mg^{-1} ml)	44.4
Carbohydrate content (%)	11 ± 0.2
Isoelectric point	3.8

Results are mean values of three replicates

concentrations of most of the metallic ions tested (Table 3), with the exception of Ag^{+} and Fe^{+2}, which inhibited the activity by 21 and 5%, respectively. This inhibition is a property in common with two sacharogenic amylases from *Rhizomucor pusillus* A 13.36, which are inhibited 88 and 46%, respectively [29]. The presence of 1.0 mM of all metallic ions slightly increased the enzyme activity. It was interesting that 1–30% NaCl did not inhibit the activity glucoamylase (data not shown), since the use of this enzyme in processes at high salt concentrations might decrease contamination risks.

Table 4 shows the glucoamylase activity in the presence of organic solvents. The glucoamylase remained completely active in the presence of 10% methanol and acetone, and in the presence of isopropyl alcohol and ethanol (10%) the enzyme maintained a high activity (86%), as compared to the control.

There are few reports with respect to the effect of organic solvent on fungal enzymes, although the effects on a prokaryotic enzyme have recently been reported [9]. Some prokaryotes are able to grow in the presence of organic solvents, and the amylases produced by these organisms are organic-solvent-stable. Organic solvents may affect the conformation of the enzyme by displacement of water molecules either on the surface or at the catalytic site [17]. Glycosylation in eukaryotic enzymes has a stabilizing function and may have a protective effect on the enzyme catalytic site against conformational change caused by organic solvents. The activity in organic solvents observed by the glucoamylase from *A. niveus* may be of industrial interest, since both acetone and ethanol are used as precipitants for the concentration of commercial enzymes. Furthermore, the addition of ethanol can also improve the overall economics of a process by reducing bacterial contamination.

Table 3 Effect of metal ions, EDTA and β-mercaptoethanol in glucoamylase activity

Ions	Relative activity (%)	
	1 mM	10 mM
$CaCl_2$	112(±0.4)	128(±0.2)
$BaCl_2$	112(±0.2)	106(±0.4)
NH_4F	105(±0.5)	112(±0.5)
NaBr	122(±0.3)	109(±0.3)
KH_2PO_4	115(±0.2)	106(±0.3)
$MnCl_2{\cdot}4H_2O$	115(±0.2)	88(±0.2)
$MgCl_2{\cdot}6H_2O$	111(±0.2)	97(±0.4)
NH_4Cl	124(±0.4)	118(±0.3)
$CuCl_2$	128(±0.5)	115(±0.5)
KCl	105(±0.4)	95(±0.1)
$AgNO_3$	79(±0.4)	51(±0.1)
$Fe_2(SO_4)_2$	95(±0.3)	52(±0.3)
$ZnCl_2$	114(±0.2)	88(±0.4)
$CoCl_2{\cdot}6H_2O$	116(±0.4)	122(±0.5)
$NaH_2PO_4{\cdot}H_2O$	117(±0.5)	117(±0.5)
β-Mercaptoethanol	114(±0.4)	100(±0.4)
EDTA	100(±0.3)	94(±0.4)
NaCl	135(±0.4)	105(±0.3)

Results are mean values of three replicates. Control (660 U/mg protein) was considered as 100%

Table 4 Effect of organic solvents in the glucoamylase activity

Solvent	5%	10%	Polarity indicate
Control	100	100	10.0
Ethyl acetate	79(±0.1)	0	4.3
N-Butyric alcohol	100(±0.2)	30(±0.2)	3.9
Isopropyl alcohol	100(±0.4)	86(±0.1)	9.3
Ethanol	100(±0.4)	86(±0.2)	5.2
Formaldehyde	53(±0.1)	0	–
Methanol	100(±0.3)	100(±0.3)	6.6
Acetone	116(±0.4)	100(±0.2)	5.4

Results are mean values of three replicates

Dichroism circular and amino acid sequence analysis

The far ultraviolet circular dichroism spectra of the glucoamylase purified from *A. niveus* (Fig. 4) presents a minimum at 207, a pronounced shoulder at 220 nm and a maximum at 192 nm. These features of the spectrum indicate that the purified protein possesses high α-helical content, and an estimate of the secondary structure content of the protein yields values of 33% α-helix, 17% β-sheet and 50% disordered coil, and indicates that the purification protocol yielded protein with native-like secondary structure.

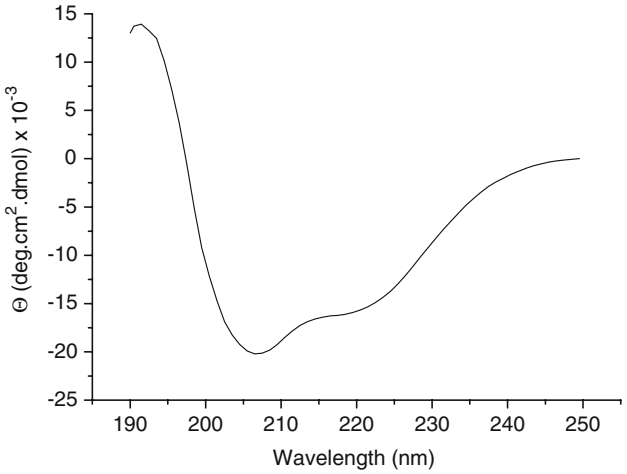

Fig. 4 Far-UV CD spectra of the glucoamylase purified from *A. niveus*. The molar ellipticity (Θ) was calculated assuming that the *A. niveus* glucoamylase presents a high degree of sequence similarity with glucoamylases from other *Aspergillus* species. See "Materials and methods" for further experimental details

Table 5 N-Terminal sequence analysis of purified glucoamylase of *A. niveus* and comparison with other *Aspergillus sp* glucoamylases

Fungi	Sequence and position	Homology (%)
A. terreus	SPRAL (299–303)	100
A. niger	SPRAL (295–299)	100
A. ficcum	SPRAL (295–299)	100
A. awamori	SPRAL (294–298)	100
A. kawachi	SPRAL (294–298)	100
A. shirousami	SPRAL (294–298)	100

SPRAL corresponds at Ser, Pro, Arg, Ala and Leu, respectively

Tryptic amino acid sequence analysis of glucoamylase from *A. niveus* was Ser-Pro-Arg-Ala-Leu (SPRAL) showing 100% identity with glucoamylases from *A. niger, A. kawachi, A. ficcum, A. terreus, A. awamori* and *A. shirousami,* as shown in the Table 5.

The secondary structure content estimate of the protein was based on the assumption that the protein shares a high degree of amino acid sequence similarity with the glucoamylases from other *Aspergillus* species, and the tryptic peptide sequence is consistent with this. The number of amino acids used to calculate the molar ellipticity was therefore taken to be 614 for the full length mature protein, which is consistent with the molecular mass of the protein after PNGase F treatment. Analysis of the full length amino acid sequence of the glucoamylases from different *Aspergillus* species reveals that the N-terminal region of the proteins (residues 36–441) shows high similarity ($E = 1e^{-115}$) with proteins from the glucosyl hydrolase family 15 (pfam00723), and the C-terminal regions (residues 546–633) are highly similar ($E = 3e^{-28}$) to the starch binding

domain family (pfam00686). The three-dimensional structures of both the glucosyl hydrolase region and the starch binding domain have been determined separately from *A. awamori* (PDB code 3GLY, [1]) and *A. niger* (1KUM, [31]), respectively, and together include 576 of the estimated 615 residues, thereby covering 94% of the total protein. Analysis of the total secondary structure content of these experimentally determined structures gives values of 38% α-helix, 17% β-sheet and 45% disordered coil. These values correlate well with the values 33% α-helix, 17% β-sheet and 50% disordered coil as estimated from analysis of the far ultraviolet circular dichroism spectrum of the glucoamylase from *A. niveus*.

Then, all analyzed properties and structural studies classified *A. niveus* as a potential thermotolerant fungus with high glucoamylase levels, which might have great possibilities for industrial application.

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